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A Method for Measuring Activated Factor VIII in Plasma

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Summary

A method is described which enables a quantitative measurement of the concentration of activated factor VIII (VIIIa) in plasma. Based on the ability of factor VIIIa to accelerate the activation of factor X by factor IXa, phospholipid and calcium ions, the course of factor X activation in time is measured using a chromogenic substrate. Free factor Xa is able to activate non-activated factor VIII present in a plasma sample, which increases the factor X activation velocity, and thus disturbs the measurement of factor VIIIa. Furthermore, factor Xa was found to be inactivated by serine protease inhibitors from the plasma sample. By adding surplus chromogenic substrate these reactions of factor Xa are inhibited and at the same time the rate of substrate conversion is a measure of the amount of factor Xa present. Factor X activation and amidolysis of chromogenic substrate then take place simultaneously. It is shown that under proper conditions the factor X activation velocity is linearly proportional to the factor VIIIa concentration. This causes the optical density to increase as a parabolic function of time. The concentration of factor VIIIa can be obtained from the quadratic coefficient of the equation describing the parabola. The method is specific for factor VIIIa in that the extrinsic factor X activator is shown to have no influence on the measurement of factor VIIIa in thromboplastin activated plasma. We conclude that a sensitive and reliable method for assessing factor VIIIa concentrations in plasma has been developed on the basis of simultaneous inhibition and measurement of factor Xa by a high concentration of chromogenic substrate.

Introduction

Clot formation in plasma is initiated by the serial enzymatic activation of clotting factors, which results in the appearance of small traces of thrombin (1). The explosive nature of the coagulation process is a consequence of the positive feedback reactions that these thrombin traces exert on the cofactors V (2–4) and VIII (4–9), and on platelets, in case of platelet rich plasma (10). Activated factors V and VIII boost the performance of factors Xa and IXa, whereas activated platelets provide, among other things, the negatively charged surface necessary for most coagulation reactions (11).

As the line separating thrombosis and bleeding seems to be a thin one, the importance of control mechanisms that steer the hemostatic process is obvious. In spite of the enormous progress that has been made by studies in systems with purified clotting factors, the fine tuning of hemostasis in vivo still remains a largely unsolved riddle. The initial traces of thrombin and the feedback reactions they provoke, are likely to play a crucial role in the overall process of hemostasis. Therefore, we set out to develop a method to assess directly one of those feedback reactions in plasma, viz. the activation of factor VIII.

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Traditionally, factor VIII concentrations, and also factor VIII activation in plasma were measured using coagulation assays, involving VIII deficient plasma (12–17). The occurrence of various feedback reactions made it impossible to relate, in a quantitatively reliable way, obtained values to amounts of factor VIII activated. The advent of a chromogenic substrate for factor Xa made a more direct way of determining functional factor VIIIa conceivable. The property of activated factor VIII, to accelerate the activation of factor X by factor IXa, phospholipid and calcium ions, by several orders of magnitude, was molded into a sensitive assay procedure for VIIIa (16, 17). However, feedback reactions of factor Xa on factor VIII (18–21) and factor X (22), and the inactivation of factor Xa by the antithrombin III and α1-antitrypsin present in plasma, can seriously hamper the feasibility of using the same type of assay for the measurement of factor VIIIa in plasma. On the basis of a method by Pieters et al. (4) we developed a scheme for measuring factor VIIIa in plasma, that circumvents these difficulties.

Materials and Methods

Materials. Factor Xa chromogenic substrate (CH_3OCO-D-CHG-Gly-Arg-pNA-AcOH) and thrombin inhibitor α-NAPAP (N-α-(2-Naphtylsulfonyl-glycyl)-D,L-Amidinophenyl-alanine-piperidine HCl) were obtained from Pentapharm (Basel, Switzerland). α-NAPAP in a concentration of 1 μM inhibits thrombin mediated amidolysis of factor Xa chromogenic substrate for 99.5%, whereas factor Xa is inhibited no more than 1%, where thrombin concentration is 20 nM and factor Xa concentration 0.75 nM (23). Buffer used was 50 mM Tris, 175 mM NaCl, 0.5 g/l ovalbumin, pH 7.9. All chemicals were to the highest grade commercially available.

Proteins. Bovine factors X, Xa, IXa and IIa were purified as previously described (24–27).

Phospholipid. Phospholipid vesicles (PL) used were 80 mole-% egg phosphatidyl choline and 20 mole-% brain phosphatidyl serine (Sigma). They were prepared as described by Rosing et al. (28).

Preparation of plasma and euglobulins. Plasma was prepared (29) from blood from 12 healthy male donors, 9 volumes collected on 1 volume of trisodium citrate (0.13 M). Plasma was defibrinated by mixing an aliquot of plasma with 1/50 volume of reptilase reagent (Boehringer Mannheim), letting a clot form for 5 min at 37° C and keeping the clotted plasma at 0° C for 10 min. The fibrin formed was discarded by winding on a small plastic spatula. Euglobulins were prepared from plasma by precipitation at low ionic strength, pH 5.2 (plasma diluted 1 in 20 in a 0.016 vol-% acetic acid solution), on ice for 30 min. After centrifugation (10 min, 4,000 rpm, 4° C), they were resuspended in 5 volumes NaCl-aq (9 g/l) and 1 volume trisodium citrate (0.13 M), so as to contain a concentration of factor VIII that was not lower than 75%. 10 nM factor Xa remains stable in this solution for over 1 h, in the presence of 0.1 U/ml heparin. Hemophilia A plasma was obtained from a single donor and it contained less than 1% factor VIII activity.

Human brain thromboplastin was prepared as described by Owren and Aas (30). It was subsequently centrifuged at 1,000 rpm for 5 min and stored in 50 μl aliquots at −80° C. It was thawed and diluted 1 in 18 with buffer containing 167 mM $CaCl_2$, then incubated at 37° C for 1 h, and kept at room temperature.

Experimental conditions. Final concentrations of the reactants used were: factor IXa: 100 nM, PL: 20 μM, Ca^{2+}: 5 mM and factor X: 330 nM. These are described by Wagenvoord (23) to be optimal for

factor X activation in a bovine system with human factor VIIIa. The same holds for pH (7.9) and PS:PC ratio of the phospholipid vesicles (20/80 mole/mole). All experiments were performed at 37° C.

Factor Xa assay. 300 µl from a factor X activation mixture was subsampled in 372 µl buffer containing 20 mM EDTA. This can be shown to stop factor X activation instantaneously. After addition of 28 µl of the chromogenic substrate for factor Xa, to a final concentration of 80 µM, absorbance was read kinetically for 2 min. The factor Xa concentration could be inferred from the initial linear increase in absorbance, using a linear least squares fit procedure. All mixtures that were assayed for factor Xa, contained 2.33 µM α-NAPAP, resulting in a final concentration of 1 µM, thereby blocking thrombin activity towards the chromogenic substrate for factor Xa for more than 99%.

Measurement of absorbance. All photometry was carried out at 405 nm in a sensitive dual wavelength photometer.

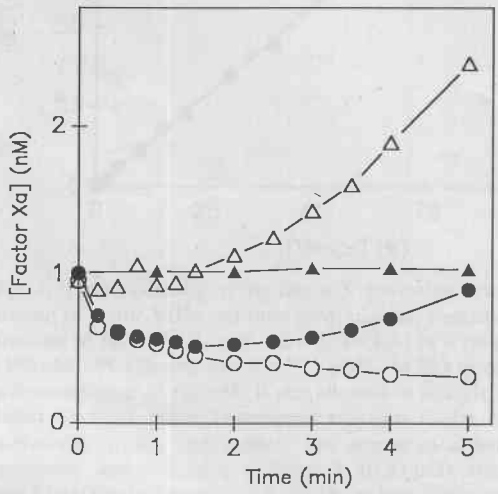

Fig. 1 Influence of factor Xa on factor X activation. To a mixture of factor IXa (100 nM), PL (20 µM), Ca^{2+} (5 mM) and α-NAPAP (2.33 µM), factor X was added to a final concentration of 0.33 µM. At 1 min, plasma (closed circles), hemophilia A plasma (open circles), euglobulin fraction (open triangles) or buffer (closed triangles) were diluted 1 in 30 in this mixture. One min later, factor Xa was added to a final concentration of 1 nM. At intervals 300 µl were taken from this mixture for assay of factor Xa, as described in the materials and methods section

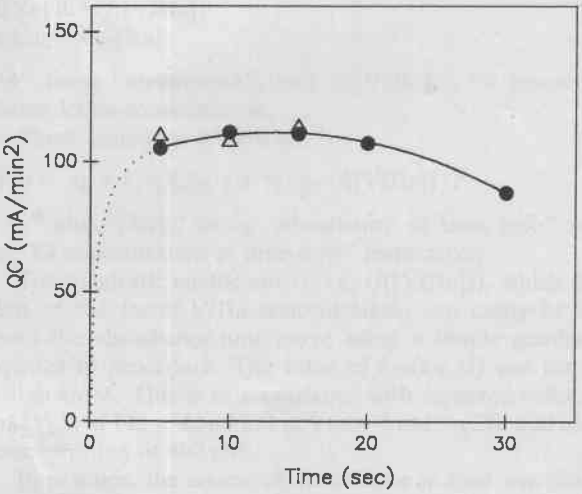

Fig. 2 Stabilization of factor VIIIa. Plasma was diluted 100 fold in a cuvette containing IXa (100 nM), PL (20 µM) and Ca^{2+} (5 mM). At 30 s thrombin was added to concentrations of 100 nM (closed circles, mean of three experiments) or 200 nM (open triangles). At various times hereafter, chromogenic substrate for factor Xa (400 µM) and α-NAPAP (1 µM) were added. The course of absorbance in time was measured upon addition of factor X (0.33 µM), 10 s later. On the vertical axis are the values of the quadratic coefficient (QC) of the equation describing the absorbance time curves ($½ \cdot k_2 \cdot f$ ([VIIIa]), see eq. III). They were obtained using a quadratic least squares fit procedure. As we were not able to determine the course of factor VIII activation during the first 5 s after thrombin addition, the curve shown is putative and plotted as a dotted line

Results

Since factor VIIIa is not an enzyme, its functional concentration cannot be measured directly by way of a chromogenic substrate. Instead, the ability of factor VIIIa to enhance factor X activation by factor IXa, phospholipid, and Ca^{2+}, must be exploited. The concentration of factor Xa can be determined chromogenically, which permits an estimation of the factor VIIIa level.

Activation of Factor VIII by Factor Xa

It is long known that factor Xa is able to activate factor VIII, be it less effectively than thrombin. Since in plasma, the medium for which we intended to develop the factor VIIIa assay, both activated and non-activated factor VIII may be present, this feature of factor Xa is a potential hazard to the reliability of any method.

The effect of factor Xa on the time course of factor X activation was investigated by adding a small quantity of factor Xa to a mixture containing factors IXa and X, PS:PC vesicles, Ca^{2+} and non activated plasma. Thrombin mediated activation of factor VIII was prevented by the presence of the thrombin inhibitor α-NAPAP. In case of factor Xa activation of factor VIII, this will result in the formation of complete tenase, the complex of factors IXa and VIIIa on a phospholipid surface, which leads to an increase of the factor Xa concentration. When factor Xa would not act on factor VIII, factor Xa concentration would remain unaltered.

Rather surprisingly, Fig. 1 shows an initial drop of the factor Xa concentration which is followed by an increase. When the experiment was repeated with hemophilia A plasma, and with the euglobulin fraction of normal plasma (no serine protease inhibitors), either the downward or the upward bend of the curve was found to disappear. Using buffer instead of plasma causes the factor Xa concentration to remain constant.

Thus it seems likely that factor Xa indirectly causes factor X activation by activating factor VIII, but also that factor Xa is subject to inactivation by inhibitors (ATIII, α1 antitrypsin) present in the diluted plasma. When the experiments were repeated with a high concentration of hirudin (1.25 µM) instead of α-NAPAP, a similar course of X activation was obtained (results not shown).

Both effects, factor Xa mediated activation of factor VIII and factor Xa inactivation directly affect the signal used for the determination of factor VIIIa. Because the measurement is based on the ability of factor Xa to split chromogenic substrate, adding a factor Xa inhibitor will be effective to inhibit these undesired reactions, as well as the factor Xa measurement itself.

Inhibition of Factor Xa by Chromogenic Substrate

The only "inhibitor" that can dampen the side reactions without influencing signal generation is the chromogenic substrate for factor Xa, at a concentration sufficiently above K_m. This would prevent all reactions of factor Xa but amidolysis of chromogenic substrate. It means that factor X activation by the

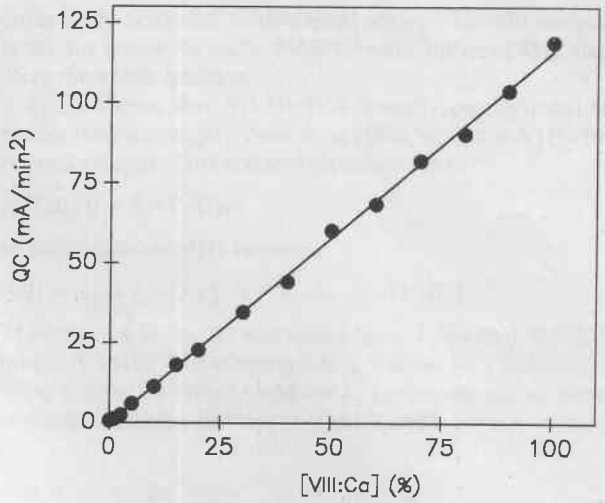

Fig. 3 Proportionality of the factor X activation velocity to the concentration of factor VIIIa. At time zero, plasma, containing various concentrations of factor VIII, was diluted 100 fold in a cuvette containing IXa (100 nM), PL (20 μM) and Ca^{2+} (5 mM). At 30 s thrombin was added, to a concentration of 100 nM. It was allowed to activate factor VIII for 15 s (until $t = 45$ s), when chromogenic substrate for factor Xa (400 μM) and α-NAPAP (1 μM) were added. The course of absorbance in time was measured upon addition of factor X (0.33 μM) after another 10 s (at $t = 55$ s). On the vertical axis are the values of the quadratic coefficient (QC) of the equation describing the absorbance time curves ($½ \cdot k_2 \cdot f$ ([VIIIa]), see eq. III). They were obtained using a quadratic least squares fit procedure. The concentration of VIII in the plasma was varied by mixing pooled normal plasma with hemophilia A plasma

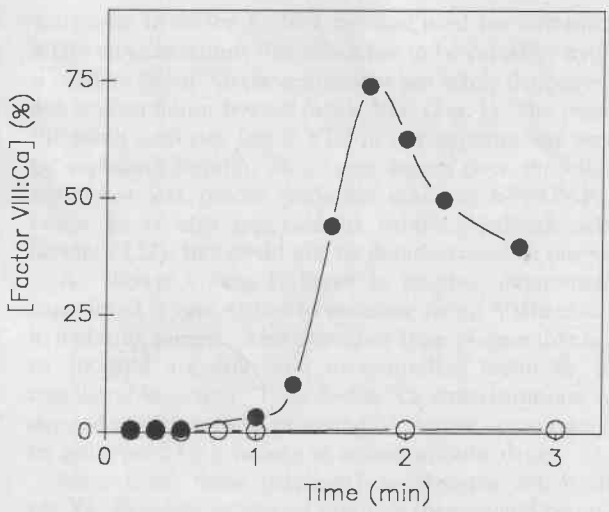

Fig. 4 Specificity of the factor VIIIa assay in thromboplastin activated plasma. 10 μl of a 1 in 18 dilution of thromboplastin in a 167 mM $CaCl_2$ solution were added to 90 μl of defibrinated plasma (final dilution of 1 in 180). At various times, a sample of this mixture was diluted 1 in 90 in a cuvette containing factor IXa (100 nM) (closed circles) or no factor IXa (open circles), PL (20 μM), Ca^{2+} (5 mM) and α-NAPAP (1 μM) in buffer, so as to obtain a final dilution of the plasma of 1 in 100. After 10 s, factor Xa chromogenic substrate was added, to a concentration of 400 μM. After another 10 s, factor X (0.33 μM) was added to initiate the activation reaction, and absorbance was measured during 2 min. Factor VIIIa concentrations are plotted as a percentage of the total factor VIII level

tenase complex and chromogenic substrate conversion are two concurrent processes.

The following equations describe these simultaneous reactions in the mixture:

$$d[Xa]/dt = f([VIIIa]) \quad (I)$$

$$dA/dt = k_2 \cdot [Xa] \quad (II)$$

"A" being "absorbance", and "$f([VIIIa])$", "a function of the factor VIIIa concentration".

These equations resolve in:

$$A(t) = A_0 + k_2 \cdot [Xa]_0 \cdot t + ½ \cdot k_2 \cdot f([VIIIa]) \cdot t^2 \quad (III)$$

"A_0" and "$[Xa]_0$" being "absorbance at time zero" and "factor Xa concentration at time zero" respectively.

The quadratic coefficient ($½ \cdot k_2 \cdot f([VIIIa])$), which is a function of the factor VIIIa concentration, can easily be obtained from the absorbance-time curve using a simple quadratic least squares fit procedure. The value of k_2 (eq. II) was found to be 170 mA/nM. This is in accordance with reported values for K_m and V_{max} of 142 μM and 230 mA min^{-1} nM^{-1} (23), and a substrate concentration of 400 μM.

In practice, the course of absorbance in time was determined using the following general scheme: A sample from the mixture being assayed was diluted in a cuvette containing IXa (100 nM), PL (20 μM), Ca^{2+} (5 mM) and α-NAPAP (1 μM). After 10 s, chromogenic substrate for factor Xa was added, to a concentration of 400 μM. Factor X activation was started after another 10 s by addition of factor X to a concentration of 0.33 μM (concentrations were obtained after all additions had been made). The course of the absorbance in time was then measured at 405 nm.

The influence of factor Xa in this system was again investigated by adding a small quantity (1 nM) of factor Xa to the factor X activating mixture. This was carried out essentially as described in the general scheme above, but with factor Xa (1 nM) added 10 s after the addition of factor X. The experiment, performed with pooled normal plasma, hemophilia A plasma, euglobulin solution or buffer, resulted in absorbance-time curves that were nearly identical (less than 3% deviation at 5 min). This indicates that all reactions of factor Xa, except for the amidolysis of the chromogenic substrate, were indeed effectively inhibited.

Standard Curve

For the concentrations used, i.e. factor IXa 100 nM, PL 20 μM and factor VIIIa less than 50 pM, the amount of complete tenase that is formed is expected to depend only on the concentration of factor VIIIa, since published apparent K_d values for factor IXa on a mixture of phospholipid and factor VIIIa are below 10 nM (21, 27). Our strategy to establish this was first to find a way to stabilize factor VIIIa, so that all factor VIII in a plasma sample could be activated without inactivation going on simultaneously. Then the concentration of factor VIIIa could be varied simply by varying the factor VIII concentration by way of mixing normal plasma with hemophilia A plasma, and adding thrombin to this mixture.

Purified factor VIIIa has been found to be stabilized by complex formation with factor IXa on a negatively charged phospholipid surface in the presence of Ca^{2+} (32). It turned out that stability of VIIIa was achieved for about 20 s when plasma was diluted first in a mixture of factor IXa, PL, and Ca^{2+}, and then activated with a large amount of thrombin (100 nM) (Fig. 2).

Fig. 2 also shows that the level of the quadratic coefficient did not increase when the double amount (200 nM) of thrombin was used, indicating that all the factor VIII had indeed been activated.

Plasma samples with different factor VIII concentrations were obtained by mixing pooled normal plasma and hemophilia A plasma in various ratios. The factor VIII in these samples was

completely activated as described above. The chromogenic substrate for factor Xa and α-NAPAP were added at 45 s, that is 15 s after thrombin addition.

Fig. 3 shows that f([VIIIa]) is linearly proportional to VIIIa concentrations ranging from 0 to 100% of factor VIIIa in pooled normal plasma. This reduces equation (I) to

$$f([\text{VIIIa}]) = k_1 \cdot [\text{VIIIa}] \qquad \text{(IV)}$$

so that equation (III) becomes:

$$A(t) = A_0 + k_2 \cdot [\text{Xa}]_0 \cdot t + \tfrac{1}{2} \cdot k_1 \cdot k_2 \cdot [\text{VIIIa}] \cdot t^2 \qquad \text{(V)}$$

The value of $\frac{1}{2} \cdot k_1 \cdot k_2$ was found to be 1.164 ± 0.011 (S.E.) mA min^{-2} % VIIIa^{-1}. Assuming 100% VIII to be 1 nM (33, 34), and using a value of 170 mA/nM for k_2 (as previously mentioned), k_1 is estimated to be 137 $\text{min}^{-1} \cdot$ (nM Xa/nM VIIIa).

Specificity of the Assay

Aspecificity of the factor VIIIa assay can be introduced at two levels: conversion of chromogenic substrate by anything other than factor Xa generated in the assay mixture, and activation of factor X in the assay mixture by anything other than the IXa-VIIIa complex. The first means of aspecificity happens not to have an influence on the assay at all, since it only affects the linear coefficient of the quadratic equation describing the absorbance time curves (eq. V), whereas the factor VIIIa concentration only appears in the quadratic coefficient.

A small amount of aspecific activation of factor X is of course expected to be caused by factor IXa which is not complexed to factor VIIIa. Since the assay is intended to measure factor VIIIa concentrations in a plasmatic environment, aspecific activation of factor X in the assay mixture may also come from the extrinsic factor X activator, the complex of tissue factor with factor VIIa.

The specificity of the factor VIIIa assay scheme was tested using buffer, non-activated plasma and thromboplastin (final dilution 1 in 180) activated plasma. This dilution of thromboplastin clotted uninhibited, non-defibrinated plasma in 70 s. When we employed the general scheme described above to measure factor VIIIa activity in buffer and non-activated plasma, identical control values for the quadratic coefficient of 0.6 mA/min^2 were found. Als already mentioned, this value is the result of the slow activation of factor X by IXa in the presence of phospholipid and Ca^{2+}, but without factor VIIIa.

In order to be able to determine any aspecificity in thromboplastin activated plasma, we suppressed the factor VIIIa based acceleration of factor X activation. This was done by excluding factor IXa from the factor X activating mixture, so that any factor X activation would be caused by constituents from the thromboplastin activated plasma. Fig. 4 shows that very little factor X activating activity could be detected. The absorbance values obtained in the absence of factor IXa were so low, that a quadratic curve could not reliably be fitted to the experimental absorbance-time data. Instead, we made an estimation of the maximal values of the quadratic coefficient by assuming the constant and linear coefficient to be zero, and calculating the quadratic coefficient from $\frac{1}{2} \cdot k_1 \cdot k_2 \cdot [\text{VIIIa}] = A(t)/t^2$ at t = 2 min.

Discussion

Although thrombin seems to be the only physiological activator (4), factor Xa was shown to be able to activate factor VIII in purified systems (18–21). Factor X activated in a system containing factor IXa, factor VIII, phospholipid and Ca^{2+} causes generation of factor VIIIa that in turn will accelerate the activation of factor X. In a method used for determining factor VIIIa concentrations this effect has to be carefully avoided, since it renders factor Xa concentrations not solely dependent on VIIIa but also on non-activated factor VIII (Fig. 1). The possibility that thrombin activates factor VIII in our experiments was excluded by replacing hirudin, in a large excess over thrombin, for the somewhat less potent thrombin inhibitor α-NAPAP. Although factor Xa is also reported to exhibit feedback activation of factor X (22), this could not be demonstrated in our system.

As shown in Fig. 1, there is another phenomenon to be considered if one wishes to measure factor VIIIa concentrations in a plasma sample. Anti-proteases from plasma that is diluted up to 100-fold are still able to neutralize factor Xa to a quite considerable extent. Thus factor Xa concentrations would also depend on the anti-Xa potential of plasma, a parameter that can be influenced by a variety of antithrombotic drugs.

Since these "side reactions" involve the active site of factor Xa, the obvious way to suppress them would be an inhibition of this active site. The conversion of chromogenic substrate should not be affected, however. It is shown in Fig. 2 that this could be accomplished by a high concentration of chromogenic substrate for factor Xa. Factor Xa, then, is inhibited and measured in the same time.

Now, the course of absorbance in time is the result of two simultaneous reactions, the activation of factor X, and the amidolysis by factor Xa of chromogenic substrate. The latter reaction can initially be considered to be of first order, until an absorbance value of about 0.400 O.D. units.

In case of linearity between amidolysis and factor Xa concentration, the first derivative of the absorbance-time curve, dA/dt, is a function only of the other reaction going on in the mixture, viz. factor X activation in time by the tenase complex (eq. I). In all experiments performed, with activated factor VIII ranging from 0 to 100%, factor X activation was linear in time for at least 1 min. Care was taken to use only that part of the absorbance-time curve for parameter estimation (eqs. III and V).

Lollar et al. have shown that purified porcine factor VIIIa is markedly stabilized by factor IXa, PC:PS vesicles, and Ca^{2+}, which prolong the half-life time from 7 min to about 1 h (32). As thrombin-activated factor VIII in plasma is much more unstable than its purified counterpart, so is factor VIIIa in plasma that is diluted 1 in 100 in a mixture containing factor IXa, phospholipid and Ca^{2+}. It is stable for about 25 s, but this is followed by a rapid decrease, with a 50% reduction in no more than 3 min. The question about the cause of this difference in stability of native compared to purified VIIIa remains unanswered. It seems unlikely that protein C is activated in our system, as thrombomodulin is not present in plasma. Furthermore, it is reported that loss of cofactor activity of factor VIIIa does not have to be concomitant with a major alteration of its primary structure (32). Perhaps the recently published pH dependence of the stability of porcine factor VIIIa provides an explanation (31).

The short-lived stability of factor VIIIa proved sufficient to allow complete activation of all factor VIII, and thus enabled us to reliably vary the factor VIIIa concentration in order to determine just how factor X activation is a function of factor VIIIa concentration. The observed linearity is in agreement with published apparent K_d values for the tenase complex, which are lower than 10 nM (21, 27), so that factor IXa in a concentration of 100 nM is predicted to saturate factor VIIIa in concentrations that prevail in our system (0–50 pM). Wagenvoord (23) showed that factor IXa at concentrations higher than 50 nM binds factor VIIIa optimally in a similar system.

The method we arrived at does in fact not measure factor VIIIa concentrations per se, but rather factor X activating activity. By choosing the proper conditions, it was made specific for factor-

VIIIa. All unwanted side-reactions involving factor Xa could be inhibited. Any substance present in the sample diluted in the assay mixture, that has the capability to split the Xa chromogenic substrate (e.g. Xa that would be present in plasma) will only change the linear coefficient in the quadratic equation describing the course of absorbance in time, and will therefore not influence the estimation of the factor VIIIa concentration. Factor IXa in the presence of PS:PC vesicles and Ca^{2+} ions, slowly activates factor X. As the concentration of IXa is fixed at 100 nM this will always produce the same small (control) value of the quadratic coefficient. Furthermore, factor X activation by everything but factor VIIIa from thromboplastin activated plasma, notably the extrinsic factor X activator, the complex of factor VIIa with tissue factor, appeared to be negligible (Fig. 4).

A number of reports have appeared describing assay systems of activated factor VIII in purified systems (17, 19, 20, 21). In all of those systems care should, and in general, has been taken to avoid activation of factor VIII by factor Xa. This can be done by careful selection of the concentration of the reactants and by limiting the time the reaction is allowed to proceed. However, when attempting to measure factor VIII activation in plasma an additional problem springs to life i.e. factor Xa inactivation by protease inhibitors. This is a phenomenon that cannot be dealt with by adaptation of concentrations and reaction times. It is all the more dangerous because it will not affect the calibration curve, where the large quantity of thrombin added to the diluted plasma in order to activate all factor VIII present in that sample will also titrate all available antiproteases, so that factor Xa inactivation is precluded. As shown, the present method effectively deals with both side reactions in a relatively simple way.

Because the factor VIIIa assay is specific, sensitive and has a well defined theoretical base, it should make possible a thorough investigation of the physiology of factor VIII activation and inactivation, and its role in the overall process of coagulation in a close to physiological environment.

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